Supplementary Discussion

Large-scale surveys of existing drugs that may harbor antiviral activities can significantly facilitate repositioning efforts to identify efficacious treatments for COVID-19. This work reports the high-throughput analysis of approximately 12,000 known drugs evaluated for activity against SARS-CoV-2 replication. The assay, conducted in Vero E6 cells was designed to capture multicycle replication, based upon low viral input (MOI = 0.01) and an extended endpoint measurement (72 hours post-infection). To select candidates for validation studies, compounds were ranked according to their Z-score in the primary screen replicates (**Figure 1b-d**). While the average Z′ factor of the first replicate was determined to be 0.51, the duplicate ReFRAME screen harbored a 40% reduction in dynamic range and corresponding Z′ factor (0.19). Importantly, the correlation between the two screens was high (R^2 = 0.68), but, as expected, there were compounds that were found active in replicate 1, but not replicate 2. For this reason, data from replicate 1 was weighted more heavily, with 100 compounds selected exclusively from the replicate 1 dataset. Additionally, 75 compounds were selected based on average scores between the two replicates, 75 compounds were selected that were only found to be highly active in set 2, while the 48 remaining compounds were selected based on inclusion in one of the enriched GSEA categories (**Figures 2a and ED2**).These selected compounds were tested in an orthogonal assay that directly measures viral replication in contrast to the indirect measurement of replication assessed by CPE.

As noted in the main text, the immunostaining endpoint utilized in the validation screen enabled the separation of molecules that function to block CPE (i.e. cell death) from those with direct effects on replication. In addition, these validation assays were conducted employing lower drug concentrations than were utilized in the original screen (5 μM). Thus, these more stringent conditions likely removed molecules that either function to block viral-induced cell death or only function at high concentrations, both of which are unlikely to be useful in a therapeutic setting. The introduction of the described stringencies during the validation step, as well as false positive activities from the HTS assay, likely account for confirmation rates observed at this step of the analysis.

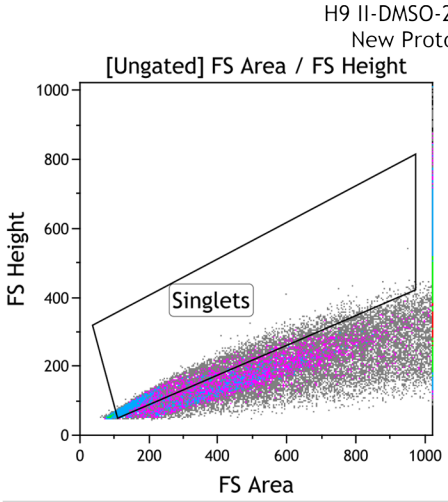
Importantly, the secondary (validation) assay was found to be most robust at a 24-hour timepoint using an MOI of 0.75, in contrast to the 72 hour endpoint with a viral MOI input of 0.01 employed in the screen. This likely biased the validation screen towards the confirmation of early stage inhibitors. Consistent with this hypothesis, we find that several molecules with potent EC_{50}s were only able to inhibit replication levels to approximately 50-60% at even at high concentrations, including MLN-3897, YH-1238 and SL-11128 (**Figures 3a-b and ED6c**). While this may represent the maximal ability of these molecules to suppress viral replication, an alternative hypothesis is that these molecules work at later stages of replication. Specifically, late acting molecules will not be able to prevent the first round of detectable infection (i.e. NP synthesis after a first wave of incoming virus), but only subsequent viral spread, and thus the maximal inhibition of infection would not be expected to reach 80-100%. Analysis of potential late-stage molecules utilizing lower MOIs at later timepoint may reveal greater inhibition of infection.

One potential limitation of employing Vero E6 cells derived from African green monkeys in the HTS assay is that species-specific differences may impact the results. For example, drugs that require the human host cell machinery for processing into their active form, such as some nucleoside inhibitors, may not harbor the same potency as in human cells. Consistently, we found that remdesivir inhibits SARS-CoV-2 replication ~60-fold more potently in human cells in comparison to Vero E6 cells (**Figures ED6c and ED7**). In contrast to direct acting antivirals, the efficacies of host-targeted therapies are reliant upon the disruption of specific cellular networks that govern host-pathogen interactions during infection, and thus can be cell-type dependent. Therefore, we further investigated if the observed antiviral activities were dependent on cellular context. Importantly, we find that a significant fraction of compounds identified in Vero E6 cells also harbor antiviral activities in multiple human cell types and retain comparable potencies (**Figures 3a, 5a-b, ED6c and ED7**). Thus, we conclude that although the use of Vero E6 cells in the initial screening assay may preclude the identification of certain potential antivirals, most known drugs identified in this campaign disrupt viral replication independent of cellular background.

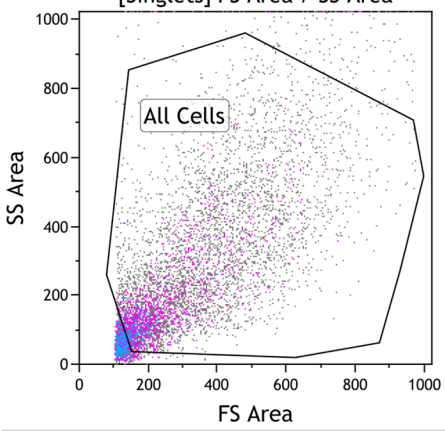
Of note, apilimod elicited some cytotoxic and/or cytostatic effects in *in vitro* cultivated cell lines at doses >100 nM (**Figure ED6a, 3b and 5a-b**). No cytotoxicity was

observed in human iPSC-derived pneumocyte-like cells (**Figure ED9c**). However, even in cells presenting cytotoxic and/or cytostatic effects, we observed nearly maximal inhibition of viral replication at 100 nM, and the selectivity index (CC_{50}/EC_{50}) of the compound was determined to be 108, and thus we conclude that the observed impact on cellular viability or growth is independent of its antiviral activity.

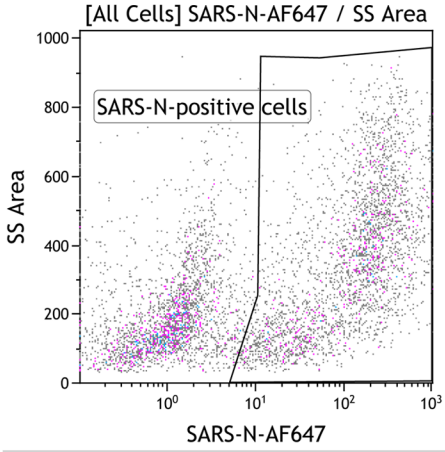
Supplementary Figure S1



Gate	Number	%Gated
All	40,361	100.00
Singlets	6,938	17.19



Gate	Number	%Gated
All	6,938	100.00
All Cells	5,624	81.06



Gate	Number	%Gated
All	5,624	100.00
SARS-N-positive cells	3,020	53.70

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